Isolation of a cDNA for a novel 120-kDa GTP-binding protein expressed in motor neurons in the salmon brain

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Abstract **We isolated a cDNA encoding a novel protein with GTP-binding motifs from the salmon brain. The cloned 5.5-kb cDNA encoded 971 amino acids, which showed limited homology with members of large GTP-binding proteins, such as dynamin, MX protein and VPS1. By in vitro translation analysis, the size of the encoded protein was estimated to be 120 kDa. The recombinant protein including the putative GTP-binding domain was shown to bind to GTP in vitro. mRNA was strongly expressed in the brain, ovary and skeletal muscle. In the brain, expression of the mRNA was observed specifically in motor neurons, in nucleus oculomotorius, in nucleus valvulae lateralis, in the medulla oblongata and in the spinal cord. To determine the cell biological significance of the encoded protein, we transfected CHO cells with wild type or a putative constitutive active mutant of the mG$_{120}$ (120-kDa G protein expressed in motor neurons) and examined morphological changes. However, no clear changes were observed. The functional role of this novel motor neuron-specific large GTP-binding protein remains to be elucidated.**

Key words: G protein; Motor neuron; Salmon; Dynamin; In situ hybridization

1. Introduction

The GTP-binding proteins are known to play roles in various cellular processes, and have a consensus motif of GTP-binding elements. They are classified into three major families by size. The first one is an α subunit of heterotrimeric GTP-binding protein, such as Gαs, Gαo, Gαi, and Gαq. They transmit signals from the metabotropic receptors or transducins to various effectors such as adenylyl cyclase and phospholipase. The second family is a small GTP-binding protein of approximately 21–28 kDa. More than 50 members of this family have been found, including Ras, Rho, Rab and Ran. They are known to play roles in cell division, regulation of cytoskeleton, vesicle transportation, and protein transportation to nuclei. The third one is a family of approximately 70–100 kDa, which we designate 'large GTP-binding protein' here. Members of this family include MX protein of vertebrates whose expression is induced by interferon [1], MGM1 protein of yeast [2], VPS1 protein of yeast which is required for the sorting of soluble vacuolar proteins [3], SRP54 protein which functions as a signal-recognition particle [4], *shibire* protein of *Drosophila* [5], and dynamin which bundles microtubules in vitro and is well known to have a role in coated-synapse vesicle formation [6,7].

We constructed a cDNA library from the salmon brain and isolated a clone that encodes a protein with a GTP-binding motif. The encoded protein (971 amino acids, 120 kDa) was larger than any other GTP-binding proteins isolated so far. The most striking feature of the isolated clone was that it is specifically expressed in the motor neurons in the brain stem.

2. Materials and methods

2.1. Isolation of cDNA clone

Poly(A)$^+$ RNA was isolated from brains of masu salmons (*Oncorhynchus masou*), and a unidirectional cDNA library was constructed as described previously [8,9]. We initially tried to isolate from this cDNA library a gonadotropin releasing hormone (GnRH) receptor by expression cloning method using *Xenopus* oocytes. At the final stage of the screening, we determined the partial DNA sequence of the candidate clones due to the seasonal problem of the oocytes. The presence of a cDNA encoding a protein with a GTP-binding motif was noticed, and the full sequence was determined as described previously [9].

2.2. In vitro translation

cRNA transcribed from the cDNA clone was translated in reticulocyte lysate with biotin-containing amino acids. In vitro translation was done using a biotin in vitro translation kit (Boehringer Mannheim). The product was loaded on SDS-PAGE and transferred to a filter membrane. Transferred protein-containing biotin was visualized by incubating with the streptavidin-HRP and ECL system. For negative control, mock reaction was done in the absence of the cRNA.

2.3. GTP-binding assay

Recombinant protein was prepared as follows. Two primers were prepared to amplify from Asn-293 to Lys-505 by polymerase chain reaction (PCR). The *Bam*HI site and *Eco*RI site were attached at the 5′ and 3′ end respectively. The PCR was done using low error Taq polymerase KOD (Toyobo). The PCR product was subcloned into pTrcHis-B vector (Invitrogen) in frame, and transformed into DH5αF′ bacteria. The recombinant protein was induced by adding 0.2 mM isopropyl thiogalactopyranoside (IPTG). For negative control, a culture without IPTG application was prepared. The bacteria were collected by centrifugation, and lysed by 6 M guanidinium buffer and sonication following the instruction of the Express System (Invitrogen). The supernatant was dialyzed by 10 mM Tris (pH 8.0), 0.1% Triton X-100 solution, and the precipitate in the dialysis tube was removed by centrifugation. The supernatant was analyzed by SDS-PAGE. These two samples (with or without IPTG induction) were used for in vitro GTP-binding analysis [10,11]. Briefly, 2.5 μg of the samples were incubated with isotope-labeled GTP ([γ-^{32}P]GTP) at 30°C in the reaction buffer [11] with 1 mM L-α-dimyristoyl phosphatidylcholine for 1 or 2 h, and trapped on nitrocellulose filter by aspiration. The filter was then rinsed to remove the unbound GTP. The radioactive count which was trapped in the filter was measured by scintillation counting. As a zero time control, which indicates non-specific background binding, the mixture was trapped on the filter immediately after mixing.

2.4. Northern and in situ hybridization analysis

Expression of RNA in various tissues was determined by Northern hybridization as described previously [12]. The entire 5.5-kb cDNA

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was labeled with ^{32}P by the random priming method and was used as a probe. The distribution of RNA in the brain was studied as described previously [12]. Probes were labeled by using a DIG labeling kit (Boehringer Mannheim). For negative control, a sense-strand probe was used.

2.5. Mutagenesis and transfection

Point mutants were made by the Sculptor in vitro mutagenesis system (Amersham) using mutated oligonucleotide DNA primers and single-stranded template DNA. The mutations were confirmed by sequencing the primer and the surrounding regions. Wild type and mutants of mG_{120} were subcloned in PCXN2 [13], a plasmid vector for efficient expression in mammalian cells. CHO cells were transfected transiently with these plasmids by lipofectamine (Gibco-BRL). Transfected cells were identified by cotransfecting with GFP (green fluorescent protein).

3. Results

We were initially engaged in the isolation of GnRH receptor cDNA of the masu salmon (*Oncorhynchus masou*) by the expression cloning method using *Xenopus* oocytes [8]. At the final round of the screening, we could not judge reliably due to the seasonal problems of the oocytes. Instead, we determined the partial sequence of the candidates. Among the candidates, there was a cDNA encoding a protein with GTP-binding motifs. By homology search analysis of the partial sequence, the clone was judged to be a novel large GTP-binding protein. Thus, we decided to determine the whole cDNA sequence.

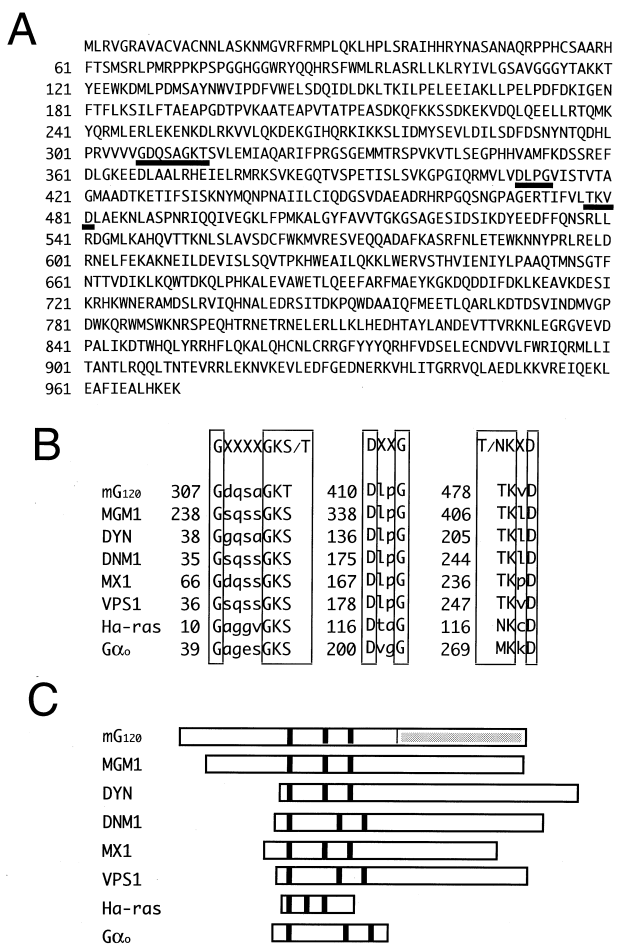

Fig. 1. Primary structure of the mG_{120} and the comparison with other GTP-binding proteins. A: Deduced amino acid sequence of the mG_{120}. GTP-binding motifs are underlined. The accession number of the sequence in GenBank is AB012720. B: Alignment of three GTP-binding elements of mG_{120}, MGM1 [2], dynamin (DYN) [6], dynamin-related protein (DNM1) [14], MX1 [1], VPS1 [3], Ha-ras [23] and Gαo. Amino acids which are recognized to be a consensus sequence are boxed. Numbers in front of the sequence motifs refer to amino acid positions in the respective proteins. C: Comparison of the overall structure of mG_{120} and other GTP-binding proteins. The bars indicate the locations of GTP-binding motifs, and the shadowed area of mG_{120} indicates the region which showed homology with myosin heavy chain.

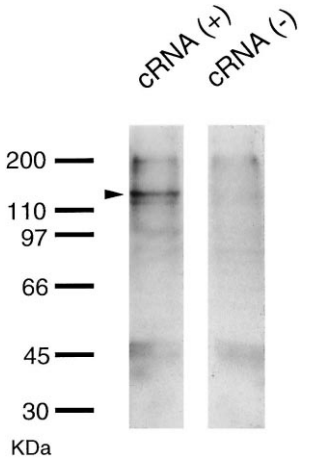

Fig. 2. In vitro translation analysis of an encoded protein by mG_{120} cDNA. SDS-PAGE analysis of protein translated in vitro by reticular cell lysate. The products of two reactions in the presence or absence of mG_{120} cRNA were compared. An arrowhead indicates the translated protein of approximately 120 kDa.

The cDNA was approximately 5.5 kb in size and contained an open reading frame that encoded 971 amino acids (Fig. 1A). It had three elements of the GTP-binding motif (GXXXXGKS/T, DXXG, T/NKXD) which are characteristically conserved in the GTP-binding protein family (Fig. 1B). In the core region including the GTP-binding motifs, the clone showed approximately 25% identity of the amino acid sequence with members of the large GTP-binding protein family such as dynamin-related protein (DNM1) [14], MX1 (interferon-induced GTP-binding protein) [1], MGM1 in yeast [2], VPS1 (vacuolar sorting protein) in yeast [3] and dynamin [6]. From these homologies and the size of the encoded protein, the isolated clone was judged to belong to the large GTP-binding protein family (Fig. 1C). However, the homology of the amino acid sequence was observed only in the core region, and there was no significant homology in other regions. By analyzing the homology of various regions with known proteins, the region after the GTP-binding motif (Thr-551 to the COOH-terminal) appeared to have limited but significant homology (22%) with the myosin heavy chain in cardiac muscle. The region of mG_{120} before the GTP-binding motif showed no significant homology with any other protein in the database.

To confirm that the cDNA actually encodes a single large protein of the expected size (120 kDa), in vitro translation analysis was done using reticulocyte lysates. A single band of 120 kDa was detected specifically in the RNA-positive lane (Fig. 2). The molecular weight of the product is compatible with the expected size from the sequence, taking the increase by glycosylation into account. This result proves that

the predicted open reading frame of 971 amino acids encoding a 120-kDa GTP-binding protein was actually used.

Although mG_{120} has a clear GTP-binding motif, it is necessary to test whether or not GTP actually binds to mG_{120}. To clarify this point, we prepared a recombinant protein of the GTP-binding domain of mG_{120} (from Asn-293 to Lys-505) as described in Section 2, and carried out an in vitro binding assay. In Fig. 3A, the purified protein of the transformed bacteria is shown. Only when protein synthesis was induced by 0.2 mM IPTG (right), a protein of the expected size (approximately 30 kDa) was observed. The slightly lower band is thought to be a processed product. As the induced recombinant protein was by far the largest population, the whole bacteria proteins were used for the in vitro GTP-binding assay. The protein sample with IPTG induction showed a clear increase in binding with time from the non-specific background binding level (Fig. 3B). In contrast, an increase with time was not observed in the protein sample without IPTG induction (Fig. 3B). From these results, it was demonstrated that GTP actually binds to the recombinant protein including the GTP-binding motif of mG_{120}. Thus, mG_{120} was proved not only structurally but also functionally to be a GTP-binding protein.

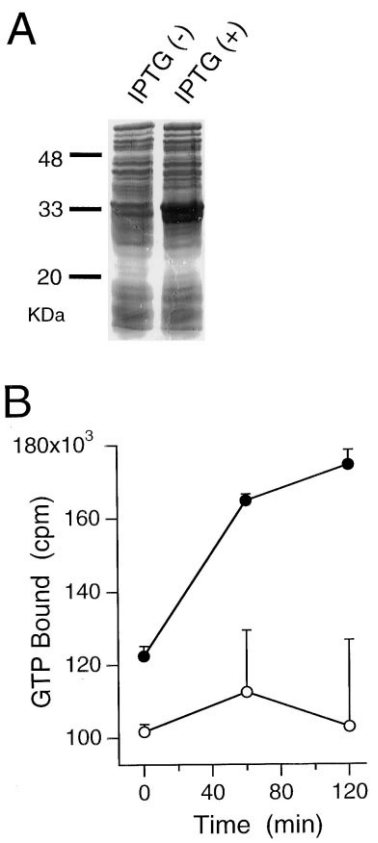

Fig. 3. Binding of GTP to the putative GTP-binding domain of mG_{120}. A: Preparation of recombinant protein which includes the putative GTP-binding domain of mG_{120} (amino acids 293–505). The transformed bacteria were cultured in the absence (left) and in the presence (right) of 0.2 mM IPTG which induces protein synthesis. The proteins were purified as described in Section 2, and were visualized by Coomassie brilliant blue staining of the SDS-PAGE. B: 2.5 μg of the whole protein samples in A (○, without IPTG induction; ●, with IPTG) were incubated with isotope-labeled GTP, and the binding was estimated as described in Section 2. The average and standard deviation of two experiments are shown.

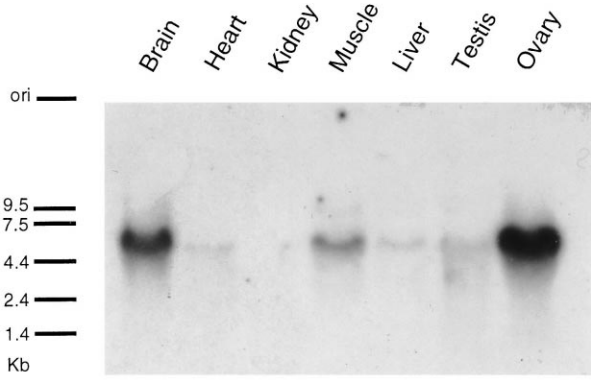

Fig. 4. Northern hybridization analysis of mG_{120} mRNA expression in various tissues. 2 μg of poly(A)$^+$ RNA from various tissues were loaded, transferred, and probed with the 5.5-kb full-length mG_{120} probe. The integrity and loading was confirmed by ethidium bromide staining of the RNA gel.

The distribution of mG_{120} mRNA was studied by Northern hybridization (Fig. 4). A single 5.5-kb band, which is identical with the length of the isolated cDNA, was detected in brain, ovary and skeletal muscle. Weak expression was detected in heart, liver and testis but not in kidney. The tissue distribution pattern was unique and differed from those of other G protein mRNAs. The identity of the sizes of the cDNA and the detected mRNA excludes the possibility that the isolated clone is an artifact in which two cDNAs are tandemly ligated.

By in situ hybridization using a digoxigenin-labeled probe, the distribution of mG_{120} mRNA in salmon brain was analyzed at the cellular level (Fig. 5). The overall view of the sagittal section is shown schematically in Fig. 5A. Expression was observed in motor neuron nuclei such as nucleus oculomotorius and nucleus lateralis valvulae (Fig. 5B). It was also observed in large neurons in the reticular formation (Fig. 5C,F), in the ventral side of the medulla oblongata (Fig. 5D,G,H) and the spinal cord (Fig. 5E). From the size and the location of the neurons, most, if not all, of the expressing cells were judged to be motor neurons. The observed unique distribution of mG_{120} mRNA differs from those of other large GTP-binding protein mRNAs.

GTP-binding proteins are known to have various functions such as the regulation of cytoskeleton and vesicular formation, and some of them are known to cause morphological changes [15,16]. By analogy, it was speculated that mG_{120} might also cause some morphological changes of the expressing cells by regulating cytoskeletal elements. To obtain a clue to the cellular function of mG_{120}, we transfected CHO cells with the wild type or one of the two mutants (Q309V or K313A) of the GTP-binding motif, and monitored the morphological changes. On the basis of previous studies of GTP-binding protein function, mutant Q309V was expected to be constitutively active [16], whereas K313A was expected to have diminished function [17,18]. However, none of the mutants or the wild type of mG_{120} caused clear morphological changes in the transfected CHO cells (data not shown).

4. Discussion

We isolated a cDNA encoding a novel member of large

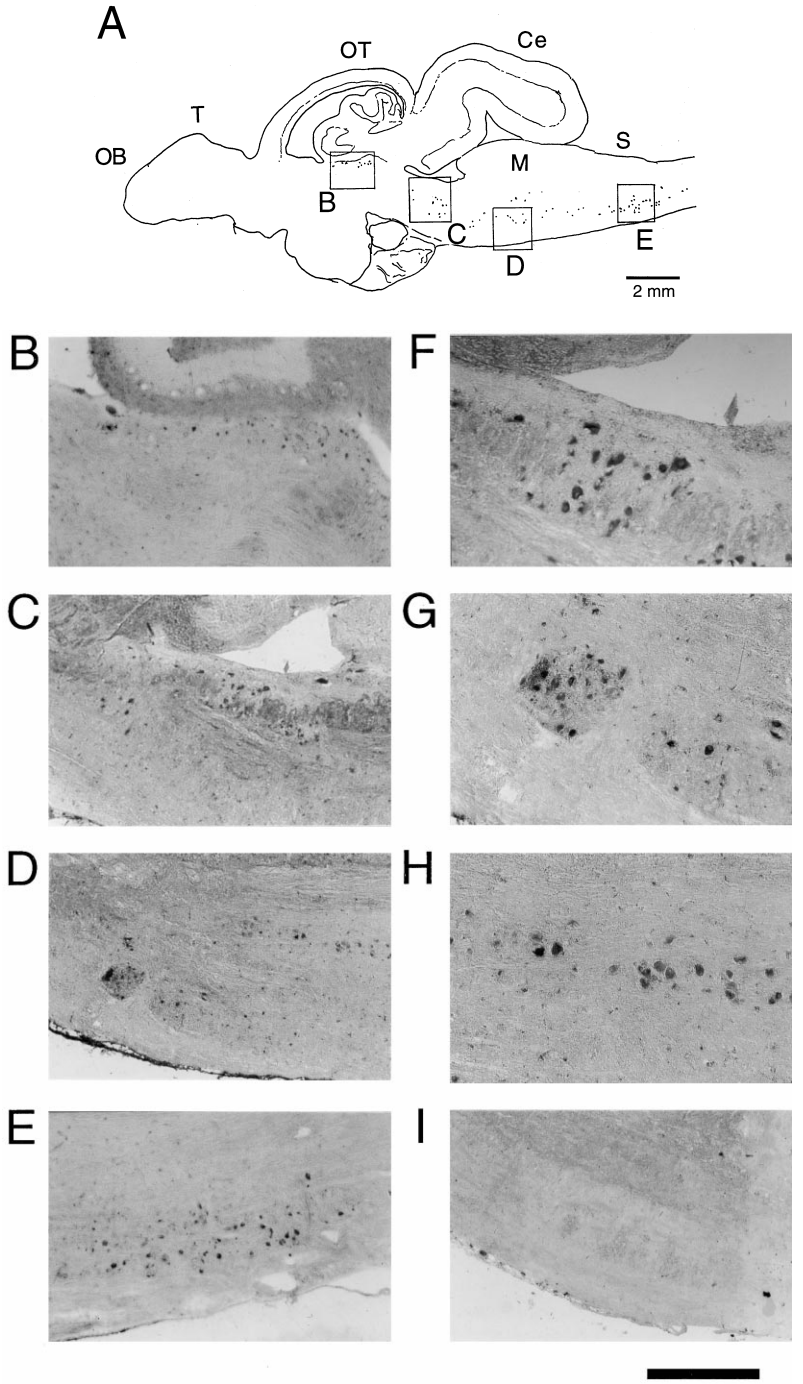

Fig. 5. In situ hybridization analysis of mG_{120} mRNA distribution in brain using digoxigenin-labeled probes. A: Schematic drawing of the sagittal section of the salmon brain to explain the expression pattern. The stainings observed are depicted by dots. The areas shown in B–E are indicated in this drawing. OB, olfactory bulb; T, telencephalon; OT, optic tectum; Ce, cerebellum; M, medulla; S, spinal cord. B–E: Sections probed by antisense probe. F: Enlarged view of C. G, H: Enlarged view of D. I: Negative control staining by a sense probe. The area corresponds to the area shown in D. Scale bar indicates 1 mm for B–E and I and 400 μm for F–H.

GTP-binding protein family (mG_{120}). It had three characteristic features, as follows.

The first point is its large size. A mG_{120} cDNA encoded a 120-kDa protein of 971 amino acids. This size is larger than any other large GTP-binding proteins cloned so far, such as MX1 (672 residues) [1], VPS1 (704 residues) [3], DNM1 (757 residues) [12], dynamin (851 residues) [6] and MGM1 (917 residues) [2].

The second point is the limited but significant (22%) homology of the COOH-terminal half with the myosin heavy chain. From this fact, it was speculated that mG_{120} has a role in the GTP-mediated regulation of cytoskeletal elements. The COOH-terminal regions of the large GTP-binding protein family are quite divergent. For example, a proline-rich region in dynamin [6], a methionine-rich region in SRP54 [4] and unique sequences without cysteine residues in VPS1 [19]

have been reported. The mG_{120} protein showed no homology with them, suggesting that mG_{120} is a totally new member of the large GTP-binding protein family.

The third and the most characteristic feature of mG_{120} is its expression pattern. In the brain, it was expressed in large neurons of nucleus oculomotorius and nucleus lateralis valvulae, which are known to be rich in motor neurons. It was also expressed in large neurons of the ventral side of the medulla and the spinal cord. The expression in motor neurons was judged by the size of the neurons and the location, and may suggest that mG_{120} has a specific role in the function of motor neurons, such as projection of long axons.

GTP-binding proteins are involved in many cellular processes. Rab proteins regulate vesicular docking and fusion [20], the Rho family interacts with the cytoskeletal actin [21] and dynamin is needed in a microtubule-associated mechanochemical function [22]. We expected that mG_{120} might also have similar effects to the expressing cells, and examined the morphological changes of the CHO cells transfected with wild type and mutants of the GTP-binding domain of mG_{120}.

The design of point mutations was based on earlier mutant studies reported as follows. The corresponding mutant of RhoD to Q309V was reported to be constitutively active due to the deficiency of GTP hydrolysis [16]. Morphological changes were observed in cells transfected with RhoD or RhoD mutants. The mutant corresponding to K313A was reported to have greatly reduced affinity for guanine nucleotides and impaired GTPase activity [17,18]. This mutant of dynamin induced striking changes in the shape and the size of the transfected cells. As none of the mutants and the wild type of mG_{120} caused apparent morphological changes in CHO cells, we could not determine the functional role of mG_{120} at present. However, from the unique structure and the distribution, mG_{120} is still thought to have significant cell biological roles. Future studies including analysis of the subcellular localization by specific antibody and identification of the interacting protein will elucidate the physiological function of mG_{120}.

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